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Genetic and epigenetic alterations in Toll like receptor 2 and wound healing impairment in type 2 diabetes patients

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ABSTRACT

Aim: Persistent hyperglycemic microenvironment in type 2 diabetes mellitus (T2DM) leads to the development of secondary complications like wound healing impairment. Proper co-ordination of innate immune system plays an integral role in wound healing. Toll like receptors (TLRs) are prominent contributors for the induction of the innate immune and inflammation response. TLR2 is an important extracellular member in mammalian TLR family and has been shown to be a potent player in the wound healing mechanism.
Methods: Expressional status of TLR2 was seen in wounds of T2DM cases with respect to the severity of wounds in 110 human lower extremity wounds. The methylation status of TLR2 promoter was also examined.
Results: Although TLR2 transcripts were downregulated in T2DM wounds compared to control, their levels tend to increase with the severity of T2DM wounds. The methylation status of TLR2 gene promoter was not significantly different among different grades of wounds in T2DM subjects. The CpG sites investigated were totally or partially methylated in majority of DFU cases.
Conclusion: TLR2 down regulation in wounds of T2DM patients compared to non diabetic patients may lead to development of non healing chronic ulcers in them.

1. Introduction

The cascade of wound healing in higher organisms generally exhibits an integration of several mutually coherent steps ranging from (i) wound homeostasis through (ii) acute inflammation, (iii) proliferation and finally leading to (iv) remodeling of the affected part (Singh, Agrawal, Gupta, & Singh, 2013a). These steps should be tightly regulated spatially and temporally as any type of imbalance may lead to non healing chronic ulcers (Whitney, 2005). The immune system, both innate and adaptive, plays an integral function in the process of wound healing, as evident by the secretion of signaling molecules like cytokines, lymphokines and growth factors (Singer & Clark, 1999; Werner & Grose, 2003). Innate immune system provides the first line of defense against foreign invaders during the process of wound healing (Park & Barbul, 2004). Signaling receptors like Toll like receptors (TLRs) are one of the most prominent contributors for the induction of the innate immune and inflammation response (Takeda, Kaisho, & Akira, 2003). TLRs are the family of transmembrane proteins, expressed on almost every immune cell like macrophages, neutrophils and dendritic cells, where their main function is to serve as a pathogen recognizing receptor (PRR) and to sense the pathogen associated molecular patterns (PAMPs) over a plethora of microbes invading during open wounds (Akira, Uematsu, & Takeuchi, 2006). After binding with these PAMPs, TLRs initiate signaling pathways that ultimately lead to activation of two main transcription factors: Nuclear factor kB (NF-kB) or type I Interferon (IFN) (Dasu & Isseroff, 2012). The TLR induced inflammation may be either agonist or antagonist of wound healing, depending upon the timing and the extent of these transcription factors which critically determine the fate of the healing wound (Dasu & Isseroff, 2012).

TLR2 is an important extracellular member in mammalian Toll family of leucin rich receptors. TLR2 is known to be a signaling receptor for many microbial products including whole gram positive bacteria, microplasma, peptidoglycan and lipoteichoic acid derived from Gram-positive bacteria (Flo, Halaas, & Torp, 2001). Anti infectious property of TLR2 is evident from the fact that the TLR2-deficient mouse strain is more prone to infection with Gram-positive bacteria *S. aureus* and shows defective clearance of spirochetes after infection by *Borrelia burgdorferi* as compared to their wild type counterparts (Kuo et al., 2013). TLR2 has an ability to form heterophilic dimers with other structurally related TLRs like TLR1 and TLR6, due to which it can recognize a wide spectrum of microbial

Conflict of interest: The authors declare that they have no conflict of interest.

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Table 1
Biochemical and demographic parameters of DFU patients (N = 102) and controls (N = 8). Data are presented as mean ± SD or as number (percentage).

Parameters	DFU (N = 102)	Control (N = 8)	p-value
Age in years; mean ± SD	54.12 ± 8.72 years	56.27 ± 3.42	0.60
BMI in kg/m^2; mean ± SD	21.45 ± 2.23 kg/m^2	23.45 ± 1.95	0.17
Duration of T2DM in years; mean ± SD	9.78 ± 4.25 years	N/A	–
Male	70 (68.62%)	5 (62.5%)	0.84
Female	32 (31.38%)	3 (37.5%)	0.87
HbA1c levels (%) (Mean, range)	10.1% (8.9%–12.5%)	N/A	–
Family history present; n (%)	13 (12.74%)	N/A	–
Nephropathy present (Serum creatinine > 1.4 mg/dl); n (%)	31 (30.39%)	N/A	–
Neuropathy present (by monofilament test); n (%)	62 (60.78%)	N/A	–
Hypertension present (systolic BP > 140 mm of Hg); n (%)	35(34.31%)	N/A	–
Retinopathy present; n (%)	11 (10.78%)	N/A	–
Dyslipidemia present (Serum cholesterol and Tgy levels > 200 mg/dl); n (%)	15(14.70%)	N/A	–
Infection present (Wound culture positive for microbes); n (%)	56(55.88%)	N/A	–
Bone involvement (Osteomyelitis); n (%)	36 (35.29%)	N/A	–

components. Alteration in the methylation pattern of TLR2 has been also described in certain epithelial diseases including carcinoma and cyst formation (Furuta et al., 2008). TLR2 has been shown to be a potent player in the wound healing mechanism. TLR2 activation after acute ischemic injury promotes the process of angiogenesis by inducing endothelial cell migration and adhesion to the wound site (Xu et al., 2013). TLR2 has been also shown to modulate the synthesis of Connexin-43 (Cx43), a gap junction protein, another potent wound healing agent (Ey, Eyking, & Gerken, 2009).

Persistent hyperglycemic microenvironment in type 2 diabetes mellitus (T2DM) leads to the development of secondary complications like cardiovascular disease, neuropathy, nephropathy, retinopathy and impairment of wound healing in patients. Wound healing impairment is a serious secondary complication of T2DM which contributes to a huge percentage of total amputations performed worldwide. As per recent data, around 25% of T2DM patients develop non healing wounds once in their life time (Singh, Agrawal, Gupta, & Singh, 2013b). The reason for this observation is that the hyperglycemic conditions in T2DM cases lead to decrease in cytokines and growth factors essential for healing of wounds (Werner & Grose, 2003). The immune response is also compromised in T2DM cases which generally leads to prolonged inflammation and unresolved infection in the wound microenvironment, thereby resulting in chronic wound which either takes a long time to heal or does not heal at all. TLRs are one of important members of immune system which is affected significantly in the T2DM individuals (Kanhaiya, Agrawal, Gupta, & Singh, 2013). Recently our group has shown that genetic and epigenetic alterations in TLRs, especially, TLR4 lead to impairment of wound healing in T2DM cases (Kanhaiya et al., 2013; Singh, Singh, Agrawal, Gupta, & Singh, 2013, 2014). In the present study we tried to look upon the expressional status of TLR2 in wounds of T2DM cases with respect to the severity of wounds. The methylation status of many CpG dinucleotides situated near the regulatory sequence in the promoter region of TLR2 gene was also examined to see the relationship of epigenetic regulation of TLR2 in the patho-physiology of diabetic wound healing impairment.

2. Materials and methods

2.1. Subjects

In this hospital based case control study, a total of 110 lower extremity wounds of different grades were analyzed, out of which 102 were diabetic foot ulcer (DFU) cases and 8 were controls. Patients were recruited from the outpatient department (OPD) clinics and operation theaters of Department of Endocrinology and Metabolism and Department of Surgery, Institute of Medical Sciences, Banaras Hindu University, Varanasi, India, during the period of July 2010 to December 2013. Patients were advised to undergo a standardized clinical and laboratory evaluation (Table 1). Family history, habits (smoking, alcoholism etc.) and disease status of each patient were recorded through a questionnaire. Previously diagnosed T2DM cases having non-healing wounds of > 4 weeks were included in the study as DFU cases. The exclusion criteria of the study included presence of co morbid disorders such as thyroid dysfunction and patients not belonging to north India. All the DFU cases had lower extremity wounds, 90% of which were located on the foot alone and the remaining 10% involved both foot and lower leg. Both the plantar and dorsal aspects of the foot were involved in majority of the cases. Age and sex matched controls were recruited by full thickness wound biopsies of post cellulitic chronic ulcers of the foot and the distal leg from the general north Indian population residing in Varanasi belonging to same ethnicity, having controlled fasting or postprandial sugar levels, no family history of T2DM. These were non-healing ulcers present for 4 weeks or more following cellulitis of the lower limb. The samples were collected at the first visit of the patients to the Diabetic foot clinic. Biopsies were taken from the wound margins during the debridement process and the histological analysis was performed to determine the cell types. Tissue samples were collected in RNAlater solution (P/N AM7020, Ambion, Inc., Austin, TX, USA) and phosphate buffer saline (PBS) for RNA and DNA isolation respectively and kept frozen at −80 °C until use. For immunohistochemical staining, samples were collected in Boiun's fixative solution and kept at room temperature. Biochemical markers, such as serum creatinine and cholesterol levels were measured using biochemical autoanalyzer (Beckman Coulter) at the clinical laboratory of the Department of Endocrinology and Metabolism. Diagnosis criteria of T2DM cases were on the basis of World Health Organization (WHO) criteria i.e. fasting plasma glucose ≥ 126 mg/dl and 2 h plasma glucose ≥ 200 mg/dl. Poor glycemic control was assessed by measuring HbA1C levels. The mean HbA1c levels of DFU subjects were 10.1% (Range 8.9% to 12.5%). Screening for neuropathy was done by taking a history of sensory loss and other symptoms such as burning sensation or paresthesias. Clinical neurological examination included assessment of the vibratory threshold perception using a 128 Hz tuning fork and assessment of pain and fine touch with a pin and 10 g monofilament respectively. The tendon reflexes and muscle power were measured in patients with sensory neuropathy. Screening for vascular involvement included detailed history of vascular insufficiency, clinical examination for signs of chronic ischemia and assessment of all lower limb pulses. A bed side hand held Doppler study was carried out in all clinically suspicious cases and ABPI (ankle brachial pressure index) of < 0.9 was considered indicative of peripheral vascular disease. Classification of wounds was done on the basis of the Wagner Grading System (Table 2). The study was approved by the Institutional Human Ethics Committee of Institute of Medical Sciences, Banaras Hindu University,

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Table 2
Distribution of DFU patients according to the Wagner's wound grading.

Wound grade	Number of patients
I	11
II	42
III	40
IV	9

Varanasi, India. Informed written consent was obtained from every participant of each group.

2.2. Semi-quantitative RT-PCR

Isolation of total RNA was done from wound biopsies using TRIzol reagent (Sigma-Aldrich) followed by DNase treatment. cDNA was synthesized from total RNA using the High capacity cDNA reverse transcription kit (Applied Biosystems, P/N 4375222) in a single step process. 4 μg (10 μl) total RNA was used as template in a total volume of 20 μl reaction, following the manufacturer's instructions. Semi-quantitative RT-PCR analysis of TLR2 was done in 102 DFU cases and 8 controls. For TLR2 expression, PCR was performed with previously described forward primer 5'-GCCAAAGTCTTGATTGATTGG-3' and reverse primer 5'-TATACCACAGGCCATGGAAAC-3' yielding 199 bp amplicons (Melmed et al., 2003). The PCR conditions were: initial denaturation step of 94 °C for 5 min followed by 30 cycles of 30 s at 94 °C, 40 s at 62 °C, 40 s at 72 °C and then a final extension step of 10 min at 72 °C. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression level was measured as an internal control to check the quality of cDNA. Primers used for GAPDH were: forward 5'-CATGAGAAGTATGACAACAGC-3', reverse 5'-AGTCCTTCCACGATAC-CAAAG-3', resulting in a 113 bp amplicon. The PCR products were scanned using a gel documentation system (Alpha Innotech). Expression of TLR2 transcript was quantized after normalization of samples using GAPDH gene, densitometrically, using AlphaImager software (version 5.5).

2.3. Quantitative Real-time PCR

A quantitative RT-PCR (RT-qPCR) experiment was also done to validate the results obtained by semi-quantitative RT-PCR. A total of 102 DFU samples of different grades on Wagner Scale and 8 controls were selected to perform RT-qPCR for TLR2 according to the manufacturer's protocol (Applied Biosystem) using previously described forward primer 5'-GGCCAGCAAATTACCTGTGTG-3' and reverse primer 5'-AGGCGGACATCCTGAACCT-3', resulting in a 67 bp amplicon (Hernandez et al., 2011). Briefly, 20 μl total reaction volume containing 10 μl SYBR Green master mix (Applied Biosystem), 0.1 μl each forward and reverse primer (10 pM/μl) and 2 μl cDNA was used in PCR using ABI 7500 instrument. PCR was performed with an initial incubation at 50 °C for 2 min, followed by 10 min denaturation at 95 °C and 40 cycles at 95 °C for 15 s, 60 °C for 1 min and 72 °C for 40 s. TLR2 expression was normalized to the mRNA levels of housekeeping gene GAPDH. ΔΔCT and relative fold change of TLR2 in DFU cases were calculated as per our previous report (Kanhaiya et al., 2013).

2.4. Immunohistochemical staining

Wound tissue samples obtained during debridement process were fixed in Bouin's solution, embedded in paraffin, and sectioned into 3 μm thick sections. 5 DFU wounds and 5 control wounds were randomly selected for the study. Anti TLR2 antibody (Catalog No. ab24192, Abcam Inc., Cambridge, MA., 1:75 in PBS) was applied to the deparaffinized sections and incubated in a wet chamber at 4 °C for 12 h. Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA) was used for immunohistochemical staining. Slides were counter-stained with hematoxylin (Himedia). Cells having brown-stained cytoplasm were regarded as positive. Similar staining time and procedure were adopted for all tissue samples. Expression patterns of TLR2 in control and T2DM wounds were done under the microscope (Nikon) using different magnifications (4, 10, 20 and 40). Documentation of acquired images was done using a calibrated digital camera system (Nikon eclipse 80i) together with the software evaluation

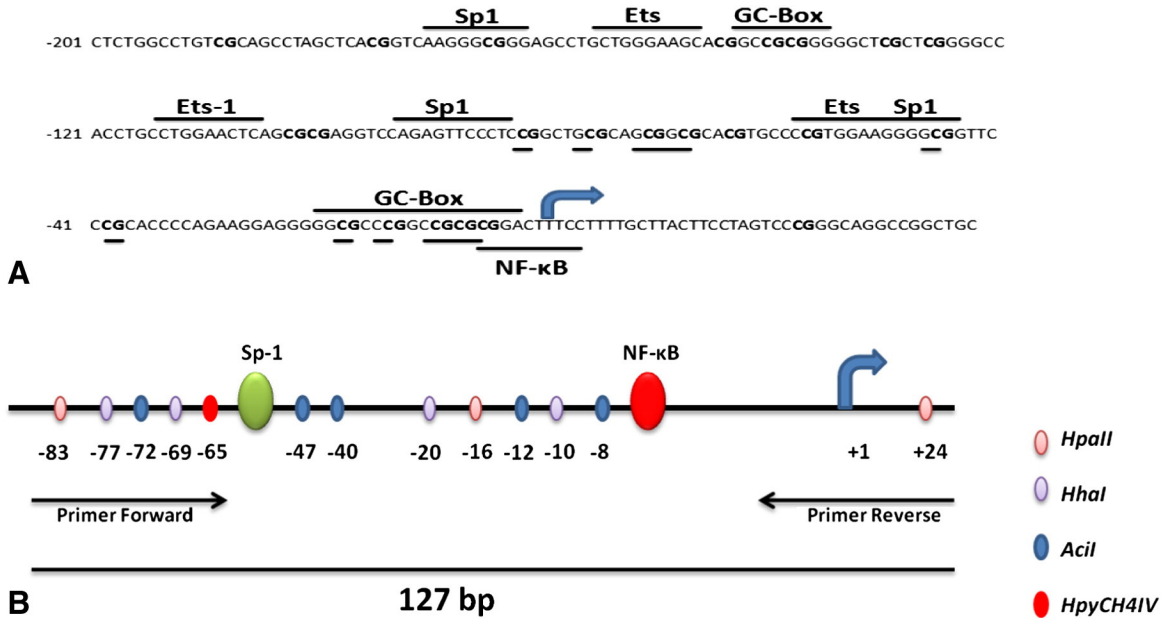

Fig. 1. TLR2 promoter. Representative diagram of the promoter of human Toll-like receptor (TLR) 2 gene is represented with (A) Genomic sequence of TLR2 promoter region ranging from −201 bp to +39 bp (Haehnel et al., 2002) with the potential sites for binding of transcription factors. Potential CpG involved in methylation is denoted in bold letters and the CpGs studied in this report are underlined. (B) Diagrammatic representation of human TLR2 promoter with small circles as restriction endonucleases sites in the promoter region and big circles as the potent transcription factors.

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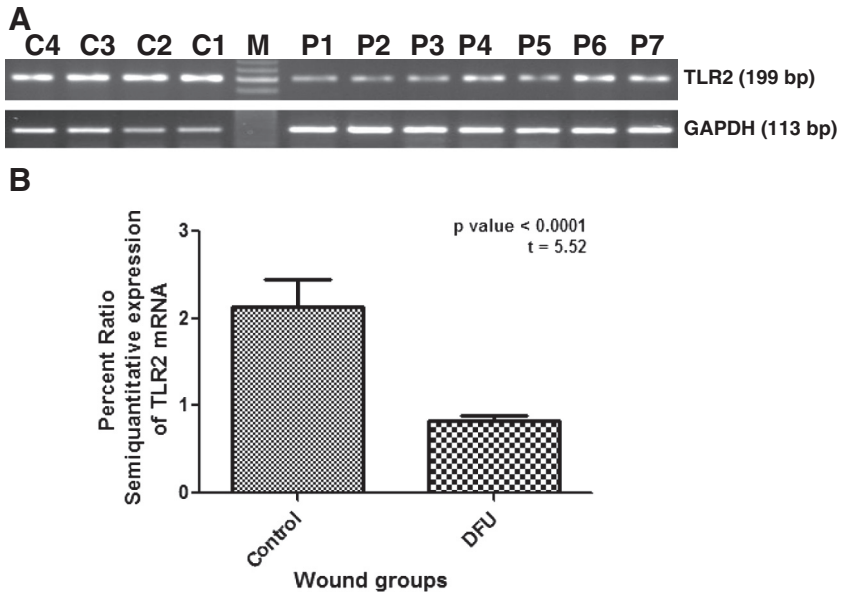

Fig. 2. Semi-quantitative RT PCR for TLR2. Semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) analysis in 102 DFU cases and 8 controls. RNA was isolated from wound samples collected during debridement of DFU patients and controls, cDNA was synthesized and RT-PCR for TLR2 and GAPDH was performed. (A) Representative picture of controls {C} and DFU {P} in each group is shown. (B) Bar graph represents the percent ratio which was calculated for the expression of TLR2 and GAPDH in Controls and DFU respectively. The percent ratio was determined by dividing the band intensity of TLR2 by GAPDH. Unpaired t test was used to check the difference between the mean values of TLR2 mRNA in DFU and control subjects. A two tailed p value < 0.05 was considered as statistically significant. Semi quantitative RT-PCR analysis shows the reduced expression of TLR2 transcripts in DFU patients (p value = <0.0001, t = 5.52, R squared = 0.22).

package (NIS Elements software). The expression density of TLR2 in wound biopsies was computed according to our previous study (Singh et al., 2014).

2.5. Immunofluorescence analysis

Tissue sections hybridized with TLR2 antibody were washed and incubated with FITC-conjugated goat anti-rabbit IgG secondary antibody (1:1000 dilutions) for 2 h in dark. The sections were washed and mounted with antifade mounting medium (Dabco® 33-LV, Sigma-Aldrich) with DAPI. Immunofluorescent sections were then assessed using a Zeiss Axiovert laser-scanning confocal microscope (Plan-Neofluar 40×) by using an FITC and DAPI specific filter set. Fluorescent images were then processed by ZEN 2012 Blue Edition software (Carl Zeiss). All of the images were captured using identical laser settings. The results were considered significant only if more than 80% of the scanned sections exhibited the observed effect.

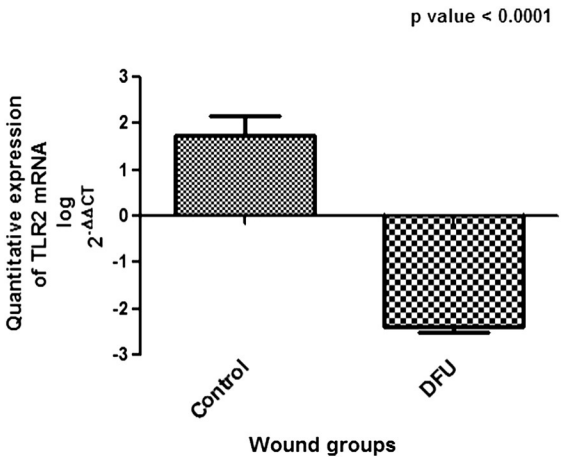

Fig. 3. Quantitative RT-PCR for TLR2. Bar graph representing Quantitative Real-time PCR analysis showing the reduced expression of TLR2 mRNA in wounds of DFU patients. Analysis was done in 102 DFU cases and 8 controls. Fold change in expression of genes was determined using the ΔΔCT method of relative quantification. Firstly normalization of the resulting threshold cycle (CT) values of the target gene was done with the CT values of the internal control GAPDH in the same samples (ΔCT = CT target − CT GAPDH). It was again normalized with the control (ΔΔCT = ΔCT − ΔCT control). The fold change in the expression was then calculated ($2^{-\Delta\Delta CT}$). The graph was plotted using $\log 2^{-\Delta\Delta CT}$. The graph clearly showed that TLR2 was decreased significantly in the wounds of T2DM cases (p value = <0.0001, t = 5.40, R squared = 0.21).

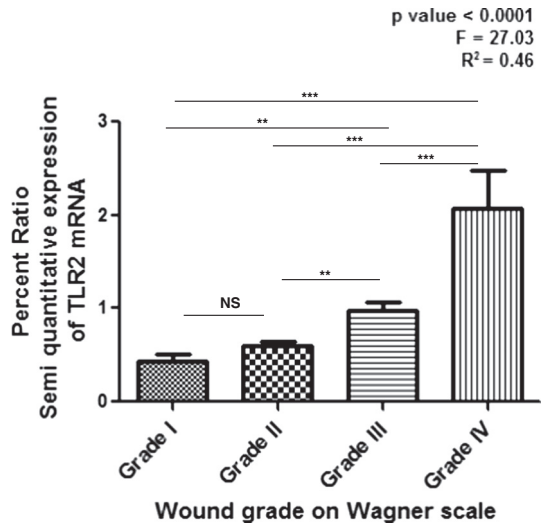

Fig. 4. TLR2 expression and wound grade. Bar graph showing the comparison of semiquantitaive TLR2 mRNA expression with wound grades on Wagner scale. A total of 102 lower extremity diabetic wounds were analyzed out of which 11 were grade I, 42 were grade II, 40 were grade III and 9 were grade IV wounds. One way analysis of variance (ANOVA) test was used to check the differences between means of TLR2 mRNA expression between the different wound grades. A two tailed p value < 0.05 was considered as statistically significant. Bonferroni's multiple comparison test was also done in order to check the statistical significance between each grade. Comparison between different grades of diabetic wounds suggested that the mRNA transcripts of TLR2 followed an increasing trend with the wound severity on Wagner's scale (p value = <0.0001, F = 27.03, R squared = 0.46). Bonferroni's multiple comparison test also indicated the significant differences of TLR2 levels between wound grades except between grade I and grade II diabetic wounds.

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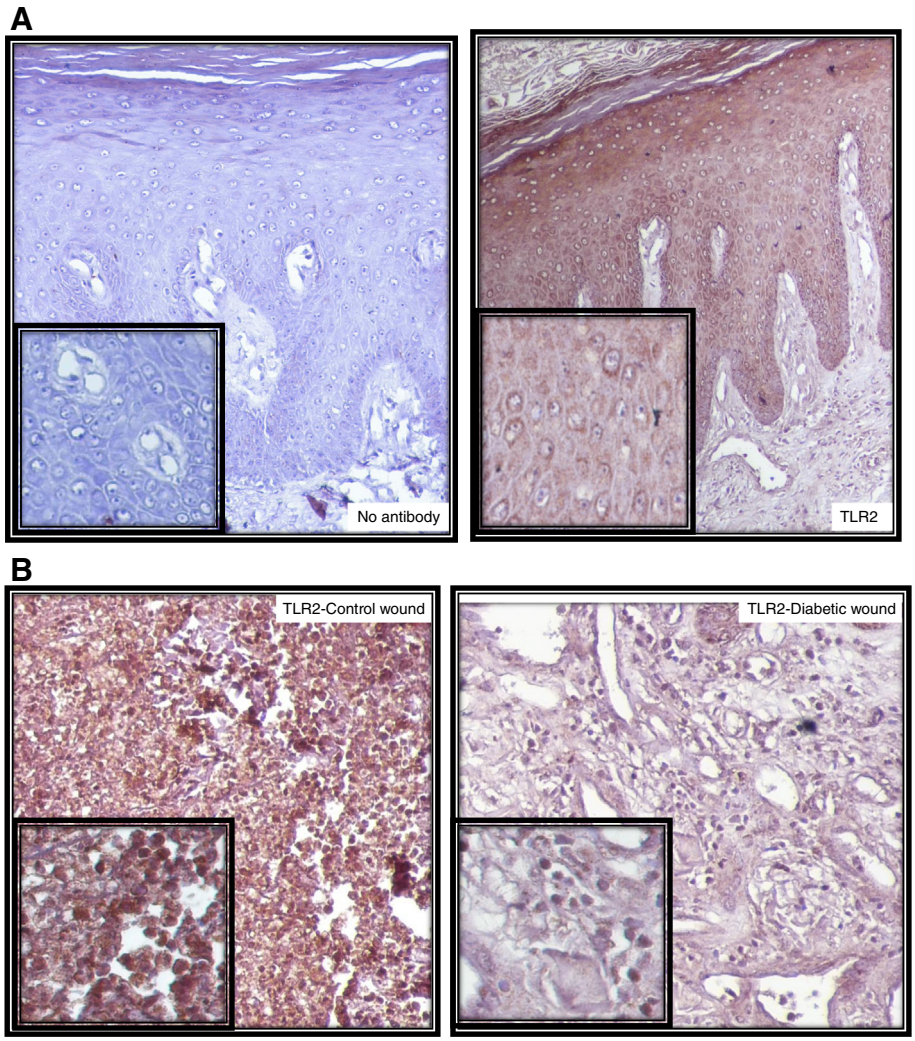

Fig. 5. IHC for TLR2 expression. Immunohistochemistry of TLR2 in wound samples (3 μm thick dewaxed paraffin sections) with insets showing detail of staining (40× magnifications). (A) The negative (left) and positive staining (right). In negative control, serum and secondary antibodies were applied but no primary TLR2 antibody was added to the staining solution to check the non specific binding of primary antibody. (B) The immunohistochemistry for TLR2 in non-diabetic control wound (left) and diabetic wound (right) which suggested significant difference of TLR2 between the wound biopsies of DFU cases and controls.

2.6. Western blot

Western blot analysis was performed for TLR2 on whole-tissue extracts of wound biopsies in 5 DFU and 5 control subjects to verify the results of IHC. About 50 μg of protein was loaded on 10% SDS-PAGE gel, was then transferred to nitrocellulose membrane and was then blocked with 5% of skim milk in TBS. For TLR2, rabbit polyclonal anti-TLR2 antibody (Catalog No. ab24192, Abcam Inc., Cambridge, MA., 1:1000 dilution) was used and then incubated with the secondary antibody linked to horseradish peroxidase. The immuno-reactive bands were visualized by the Enhanced Chemiluminescence System (Amersham Biosciences). Blots were stripped off and reprobed with an anti-GAPDH antibody. Alpha Imager 2200 software version 3.1.2 was used to quantify band density.

2.7. Methylation analysis of TLR2 promoter

Genomic DNA was isolated from wound samples using Phenol-chloroform method. The methylation status of TLR2 gene promoter was analyzed in 43 DFUs according to a previous study by De Oliveira et al. (De Oliveira et al., 2011). This PCR based approach relied on the inability of the restriction endonucleases to cut the methylated DNA. According to this approach if the cytosines are methylated, PCR amplification will produce a band equivalent to that of non-digested control samples. In contrast, if restriction enzyme cleavage at unmethylated sites induces DNA strand breaks, no band will be detected. TLR2 primers used in the methylation analysis were obtained from a TLR2 gene promoter previously reported by Haehnel et al. 2002 (GenBank accession number AC013303) (Fig. 1a) (Haehnel, Schwarzfischer, Fenton, & Rehli, 2002). The sites examined were recognized by one of the following restriction enzymes, whose activity was always blocked by CpG methylation: *AciI, HhaI, HpaII* and *HpyCH4IV* (Fig. 1b). The non-enzyme-treated DNA sample was also amplified along with enzyme-treated samples as a positive control for the PCR and for DNA loading, representing 100% of input DNA amplified after PCR. 200 ng of genomic DNA was completely digested with 4 U of restriction enzymes in 10 μl of sample containing water and buffer. After digestion 2.5 μl (50 ng) of digested DNA was pipetted into 18 μl of PCR master mix containing TLR2 forward primer 5'-GAGGTCCAGAGTTCCCTCCG-3' and TLR2 reverse primer 5'-CCGGGACTAGGAAGTAAGCA-3' yielding an amplicon size of 127 bp (De Oliveira et al., 2011). A semi-quantitative PCR technique was performed in order to compare the methylation levels of DFU samples of different grades. Intensity of the bands was calculated using software Alpha Imager. Non-digested samples were assumed to be 100% of total DNA amplification. Samples were classified into three groups: (i)

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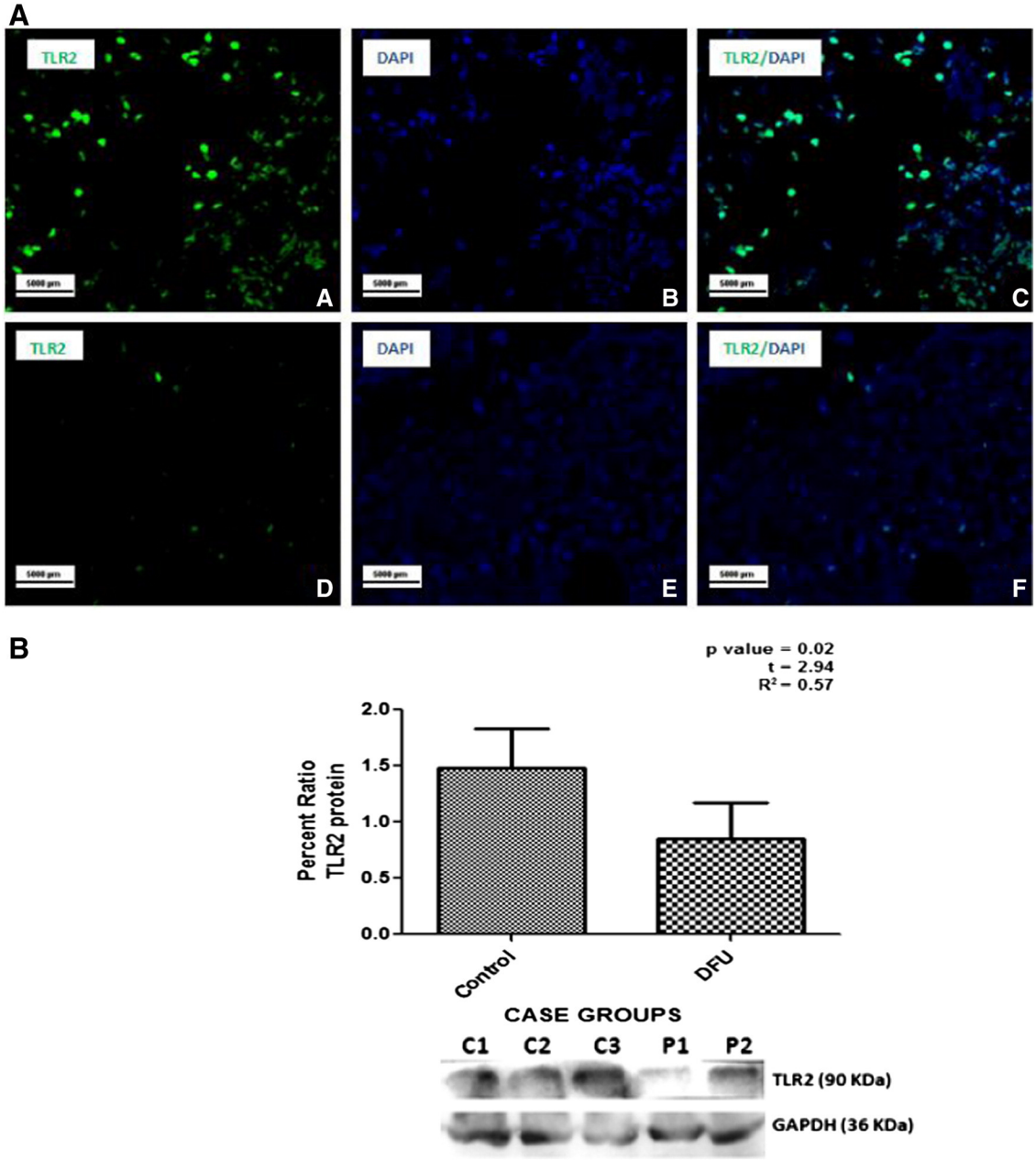

Fig. 6. (A) Immunofluorescence analysis showed lower TLR2 expression in DFU compared to control tissue. Tissue sections hybridized with TLR2 antibody were washed and incubated with FITC-conjugated goat anti-rabbit IgG secondary antibody. The sections were washed and mounted with antifade mounting medium with DAPI. Immunofluorescent sections were then assessed using a Zeiss Axiovert laser-scanning confocal microscope by using an FITC and DAPI specific filter set and similar laser settings. (A and D) Green channel showing TLR2 immunostaining, (B and E) blue channel showing immunostaining of DAPI, (C and F) showing merged image. Upper panel (A to C) shows the representative TLR2 expression in control tissue and lower panel (D to F) shows the TLR2 expression in DFU tissue. (B) Western blot assay showing that TLR2 protein expression was lower in DFU patients. Tissue samples collected during debridement process from control {C} and DFU {P} patients were homogenized, and Western blot analysis was performed for expression of TLR2 and GAPDH protein in DFU subjects and Controls. Alpha Imager 2200 software version 3.1.2 was used to quantify band density. The relative percent ratios of proteins (TLR2 vs GAPDH) were calculated. Unpaired t test was used to check the difference between the mean values of TLR2 protein in DFU and control subjects using Graph Pad Prism. A two tailed p value < 0.05 was considered as statistically significant. Bar graph showing down-regulation of TLR2 protein in wounds of T2DM patients compared to controls (p value $= 0.02$, t $= 2.94$, $R^2 = 0.57$).

methylated (band intensity equal to 100% to 75% that of undigested DNA), (ii) hemimethylated (band intensity equal to 25% to 75% that of undigested DNA) and unmethylated (band intensity less than 25% of that of undigested DNA).

2.8. Statistical analysis

The data were expressed as mean with standard error of mean as error bars. Statistical significance ($P < 0.05$) was determined with Student's t test (two-tailed) and nonparametric ANOVA followed by Bonferroni's multiple comparison test. Statistical analysis of data was performed using Graph Pad Prism 5.01 and IBM SPSS Statistics 20.0 software.

3. Results

Semi quantitative RT-PCR data suggested that the mRNA transcripts of TLR2 were significantly down-regulated in DFU subjects compared to controls (p value $= <0.0001$, t $= 5.52$, R squared $= 0.22$) (Fig. 2). The Quantitative RT-PCR results also indicated a similar down-regulation of

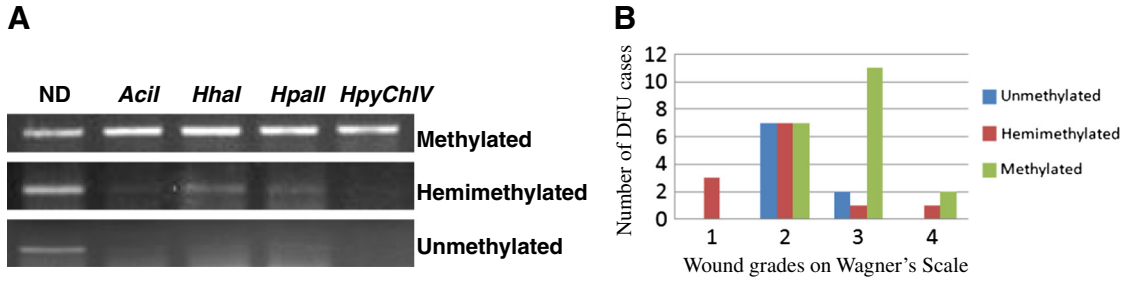

Fig. 7. Methylation status of TLR2 promoter. Methylation analysis of the TLR2 gene promoter in the different grades of DFU wounds was done using polymerase chain reaction with specific primers for TLR2 promoter yielding a amplicons size of 127 bp. (A) Representative agarose gel picture showing Methylated, Hemimethylated and Unmethylated TLR2 promoter based on the intensity of the bands obtained after the input DNA was restricted using methylation sensitive restriction endonucleases. ND indicates non-digested DNA samples corresponding to the 100% input DNA. (B) Bar graph showing methylation status of TLR2 promoter in different grades of diabetic wounds. The findings indicated that the methylation status of TLR2 gene promoter was not significantly different among different grades of wounds in T2DM subjects. The CpG sites investigated were totally or partially methylated in majority of DFU cases.

TLR2 transcripts in the DFU cases compared to controls (p value = <0.0001, t = 5.40, R squared = 0.21) (Fig. 3). Comparison between different grades of T2DM wounds suggested that the mRNA transcripts of TLR2 followed an increasing trend with the wound severity on Wagner's scale (p value = <0.0001, F = 27.03, R squared = 0.46) (Fig. 4). To determine if the difference in the message of TLR2 translates to the similar difference in protein, 5 DFU and 5 control samples were analyzed by immunohistochemistry using antibody against TLR2. Immunohistochemical expression analysis among groups also suggested significant difference of TLR2 between the wound biopsies of DFU cases and controls (Fig. 5 A and B). The results obtained were reconfirmed by immunofluorescence analysis (Fig. 6 A). A similar down regulation of TLR2 protein was found in western blot analysis (p value = 0.02, t = 2.94, R^2 = 0.57) (Fig. 6 B). Methylation analysis of TLR2 promoter indicated that the methylation status of TLR2 gene promoter was not significantly different among different grades of wounds in T2DM subjects (Fig. 7).

4. Discussion

Susceptibility to T2DM and its secondary complications like DFU is multifactorial in nature and inappropriate activation of immune system and prolonged low grade inflammation may be one of them (Singh, Agrawal, et al., 2014; Singh et al., 2014). In addition to playing a vital role in immunity, TLR2 is instrumental in coordinating tissue repair and regeneration (Ey et al., 2009). An array of current literature indicates that TLR2 is a potent modulator of wound healing in a time and expression dependent manner (Dasu & Isseroff, 2012). Wounds of T2DM patients are hard to heal because of improper integration of angiogenesis, inflammation, expression of gap junction proteins, Matrix Metalloproteinases and immune response, majority of which are also governed by TLR2 (Ey et al., 2009; Furuta et al., 2008; Singh, Agrawal, et al., 2014; Xu et al., 2013). Xu et al. (2013) have demonstrated that TLR2 promotes angiogenesis after acute ischemic injury by promoting endothelial cell migration, modulating their permeability and their invasion by lymphocytes. Another report by Ey et al. (2009) demonstrated that TLR2 is important in synthesis of Cx43 in epithelial cells which help in the formation of tight junctions and any deficiency of TLR2 signaling cause epithelial cell specific alterations in Cx43 expression. In their report Boulard, Asquith, Powrie, et al. (2010) have demonstrated that the TLR2 mediated signaling is essential for acute inflammation following microbial invasion but has no role in regulating the induction of chronic inflammation. Taking all the facts together we hypothesized that the expressional alterations of TLR2 during T2DM wounds may be one of the factors responsible for developing non healing chronic ulcers in T2DM patients. To test this hypothesis we recruited patients with non healing ulcers of different grades and analyzed the expression of TLR2 at both transcriptional and translational levels.

Our findings indicated that although TLR2 transcripts were down-regulated in T2DM wounds compared to control wounds, their levels tend to increase with the severity of T2DM wounds which was further validated by immunohistochemical analysis. This finding of ours is supported by the recent finding of Dasu and Martin (2014) which suggested that the persistent TLR2 signaling in wounds of T2DM patients may lead to hyper inflammation in wound microenvironment and may lead to development of high grade chronic wounds. Another report by Dasu et al. (2010) also supports this temporal up regulation of TLR2, in which authors have shown that the levels of TLR2 expression in day 10 wounds were high compared to day 1 wounds in spontaneously type 1 diabetic NOD mice (Dasu et al., 2010). MyD88 protein, an adapter of TLR2 mediated signaling, was also significantly higher in diabetic chronic wounds compared to normal wounds. Moreover they also showed that the wound closure is faster in diabetic TLR2 knockout mice (TLR2 −/− + STZ) compared to non diabetic mice due to a significant decrease in NF-kB activity in them. To examine the possible reason behind this down regulation of TLR2 expression, we analyzed the methylation status of 5'-proximal region of the human TLR2 gene. As per CpGPlot criteria, the promoter region of TLR2 promoter contains a typical CpG island and the region between nucleotides −281 bp and −41 marks the basic transcription machinery (Haehnel et al., 2002). The region selected for the study consisted of CpG dinucleotides present in nearby vicinity of important binding sites of transcription factors like Sp-1 and NF-κB (Fig. 1). The methylation status of TLR2 gene promoter was not significantly different among different grades of wounds in T2DM subjects. The CpG sites investigated were totally or partially methylated in majority of DFU cases.

Our present study had some limitations also. Firstly, we could not perform proteomic study on all the samples due to sample limitation (over degradation of wound tissue leading to smeared protein) and the number of control samples was less compared to DFU cases, reason being unwillingness of controls to provide tissue biopsy due to pain and fear associated in the process. Moreover, the majority of patients had intermediate grade wound (i.e. grade II and grade III) as common in Varanasi region (Kanhaiya et al., 2013; Singh, Agrawal, et al., 2014). Secondly, we used only DNA methylation event to see the epigenetic regulation of TLR2 gene. Although methylation status negatively regulates transcription of its own gene, there are other epigenetic mechanisms of gene regulation like histone modifications such as H3K9, H3K27 and H3K4 methylation apart from DNA methylation which may regulate the TLR2 gene expression which need to be investigated in the context of diabetic wound healing impairment. In conclusion we found that TLR2 down regulation at both mRNA and protein levels in wounds of T2DM patients compared to non diabetic patients may lead to development of non healing chronic ulcers in them.

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